

Inventor: Stupp et. al.

**COMPOSITION AND METHOD FOR SELF-ASSEMBLY AND MINERALIZATION
 OF PEPTIDE-AMPHIPHILES**

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/425,536 filed November 12, 2002, entitled **SELF ASSEMBLY OF MULTI-DIMENSIONAL PEPTIDE AMPHIPHILES** and the benefit of U.S. Provisional Application No. 60/425,689 filed November 12, 2002, entitled **COMPOSITION AND METHOD FOR SIMULTANEOUS SELF-ASSEMBLY AND MINERALIZATION OF PEPTIDE-AMPHIPHILES** and the benefit of International Application No. PCT/US03/04779 filed February 18, 2003, entitled **"SELF-ASSEMBLY OF PEPTIDE-AMPHIPHILE NANOFIBERS UNDER PHYSIOLOGICAL CONDITIONS"**, the contents of which are incorporate herein by reference in their entirety.

GOVERNMENT INTEREST

[0002] The United States government may have certain rights to this invention pursuant to Grant Nos. N00014-99-1-0239/P00001, DMR-9996253, and DE-FGO2-00ER45810 from respectively, the Office of Naval Research, the National Science Foundation, and the Department of Energy to Northwestern University.

BACKGROUND OF THE INVENTION

[0003] Self-assembled gels composed of peptide-amphiphile nanofibers have been described as being useful in the templated mineralization of hydroxyapatite. Peptide-amphiphiles enriched with negatively charged amino acids such as phosphoserine and aspartic acid can self assemble into nanofibers and induce hydroxyapatite crystals to grow on

the surface of the nanofiber as described by Hartgerink et al., *Science*, 294, 1683-1688, (2001). In addition to providing sites for hydroxyapatite crystal nucleation, the nanofibers also direct the growth of the hydroxyapatite crystals such that their c-axis is oriented parallel to the long axis of the nanofibers. The ability of the peptide-amphiphile nanofibers to organize and direct the growth of the hydroxyapatite crystals is reminiscent on that observed between collagen fibrils and hydroxyapatite crystals in bone.

[0004] The directed growth of hydroxyapatite crystals within organized peptide-amphiphile matrices and scaffolds is an important step toward the regeneration of mineralized materials like bone within the body.

[0005] While the preparation of oriented hydroxyapatite crystals on individual or small groups of nanofibers has been demonstrated, scaling the utility of hydroxyapatite or other minerals in bundles of nanofibers or within gels comprising nanofibers maybe limited by non-homogeneous mineralization. Non-homogeneous mineralization of nanofiber bundles or nanofiber gels results in coating of the surface nanofibers of the bundle or gel by the mineral crystals. The formed surface crystals inhibit further diffusion of mineral reagents into the interior of the nanofiber bundle or nanofiber gel and precludes formation of larger homogenous composites. In practical applications such as bone regeneration, it would be desirable that hydroxyapatite crystal growth proceed uniformly throughout the nanofiber gel matrix.

[0006] A supramolecular assembly is a material in which the constituent units or building blocks of the assembly are molecules or molecular aggregates. The interaction of the units with each other, usually by non-covalent bonding, determines the final shape and size of the supramolecular assembly. An example of a supramolecular assembly found in biological systems is α -hemolysin which is a seven protein aggregate with a non-symmetric mushroom shape. The α -hemolysin aggregate has a pore or channel that is about 16 Å in

diameter, which runs parallel to the aggregate's long axis. The aggressive human pathogen *Staphylococcus aureus* uses the asymmetric nature of α -hemolysin to implant its stem into the hydrophobic compartment of cell membranes and the hydrophilic nature of the α -hemolysin's mushroom cap to stabilize it in the extracellular space. It is through α -hemolysin's pore channel that RNA macromolecules from the *Staphylococcus aureus* pathogen can invade human cells. Synthetic supramolecular assemblies could be designed and synthesized to mimic the action of α -hemolysin's channel pore for drug delivery or other cell therapies.

[0007] The amino acid sequence IKVAV has been identified in other contexts as important for neuron growth and development. Self assembly of peptide-amphiphiles with the IKVAV sequence have been reported. These peptide-amphiphiles may facilitate neuron growth and development in supramolecular structures formed by these peptide-amphiphiles. One feature of peptide-amphiphiles having a hydrophobic alkyl tail and the IKVAV amino acid sequence in the peptide head group is that peptide-amphiphile has more than one amphiphilic moment. The peptide sequence of these and other peptide-amphiphiles can be further modified by covalent attachment of ligands or peptide sequences that can interact with various types of cells. For example, the peptide sequence Arg-Gly-Asp (RGD) occurs in fibronectin and has been found to play an important role in integrin-mediated cell adhesion. Inclusion of the RGD peptide sequence ligand into a suitable peptide-amphiphile is expected to promote cell growth and direct templated mineralization of self assembled supramolecular structures of such peptide-amphiphiles under the proper conditions. Self assembled peptide-amphiphiles are known to direct the mineralization of hydroxyapatite on the surfaces of nanofibers formed from these peptide-amphiphiles. The peptide portion of these peptide-amphiphiles can also comprise amino acid groups like cysteine, which are capable of forming

disulfide bonds between adjacent peptide-amphiphiles, and also glycine which provides flexibility to the peptide portion of the molecule.

[0008] It will be appreciated by those skilled in the art that there is a need to be able to form self assembled supramolecular structures from peptide-amphiphiles having more than one amphiphilic moment in order to take advantage of the unique cell growth, molecular transport, and templating functions that these and other related peptide sequences provide. It will also be appreciated that the self assembly occur in physiologically benign conditions of temperature, ionic strength, and pH. For the foregoing reasons, there is a need in the art to make supramolecular assemblies from multi-dimensional peptide-amphiphiles.

[0009] The present invention is directed to amphiphilic molecular compositions having more than one amphiphilic moment and also to supramolecular composition comprised of such amphiphilic molecules. More specifically, the present invention is directed to peptide-amphiphiles compositions having more than one amphiphilic moment and to supramolecular compositions comprised of such peptide-amphiphiles which self assemble in the presence of cations.

[0010] Preferred embodiments of the present invention may be useful for cell growth, molecular transport, and templating functions, especially if the self assembly occurs under benign conditions.

[0011] Homogeneously, or substantially homogeneously, mineralized self assembled peptide-amphiphile nanofibers are desirable. Homogenously mineralized materials with the mineral crystals preferentially oriented by the self assembled peptide-amphiphile nanofibers are also desired. Finally, preparing such materials under substantially neutral or physiological conditions is also desirable.

SUMMARY OF THE INVENTION

[0012] One embodiment of the present invention is directed to a composition for material formation on self assembled peptide-amphiphiles comprising at least one ionically charged species of peptide-amphiphile and at least one salt providing at least one ion from the material to be formed and having the same signed ionic charge as the peptide-amphiphile. Alternatively, the composition may be a solution of at least one species of peptide-amphiphile wherein the species of peptide-amphiphile chelates one or more ions of the material to be formed. Charged and chelating peptide-amphiphiles may also be combined to form compositions for self assembly and material formation.

[0013] The present invention is directed to a composition for material formation on self assembled peptide-amphiphiles comprising at least one ionically charged species of peptide-amphiphile; and at least one salt providing at least one ion from the material to be formed and having the same signed ionic charge as the peptide-amphiphile.

[0014] The invention is also directed to a method of making materials on self assembled peptide-amphiphiles, the method comprises preparing a first solution with at least one ionically charged species of peptide-amphiphile and at least one salt providing at least one ion from the material and having the same signed ionic charge as the peptide-amphiphile. A second solution is prepared with an ion from the material and having opposite signed ionic charge to the peptide-amphiphile in the first solution. The first and second solutions are mixed to cause self-assembly of the peptide amphiphile nanofibers and to form the material substantially on the surfaces of the peptide-amphiphile nanofibers throughout the nanofiber gel.

[0015] The present invention is directed to a composition useful for making homogeneously mineralized self assembled peptide-amphiphile nanofibers and nanofiber gels. The composition is generally a first solution comprised of a soluble positively or

negatively charged peptide-amphiphile and a soluble salt containing an ion from the mineral. The sign of the charge on the ion in the solution is the same as sign of the charge on the peptide-amphiphile. Mixing this first solution with a second solution containing a dissolved counter-ion of the mineral and/or a second oppositely charged peptide amphiphile, results in the rapid self assembly of the peptide-amphiphiles into a nanofiber gel with templated mineralization on the nanofibers of the salt ions from the solution. Templated mineralization of the initially dissolved mineral cations and anions in the mixture can occur with preferential orientation of the mineral crystals along the fiber surfaces within the nanofiber gel.

[0016] One advantage of the present invention is that it results in homogenous growth of the mineral throughout the nanofiber gel. Another advantage of the present invention is that the nanofiber gel formation and mineralization reactions occur in a single mixing step and can occur under substantially neutral or physiological conditions. These homogeneous nanostructured composite materials are useful for medical applications especially the regeneration of damaged bone or teeth in mammals. Non-medical applications of the present invention include the manufacture or coating of hard surfaces on substrates.

[0017] In another embodiment of the invention, the composition comprises one or more peptide-amphiphile species having different peptide sequences.

BRIEF DESCRIPTION OF THE FIGURES

[0018] Various aspects and applications of the present invention will become apparent to the skilled artisan upon consideration of the brief description of the figures and the detailed description of the invention which follows.

[0019] Figure 1 illustrates the chemical structure of a peptide-amphiphile $C_{15}H_{31}C(O)-CCCCGGS(P)RGD-COOH$.

[0020] **Figure 2** illustrates a transmission electron micrograph (TEM) of a homogeneously mineralized nanofiber gel sample with oriented hydroxyapatite mineral growth.

[0021] **Figure 3** is a schematic drawing of the chemical structures of the peptide-amphiphiles $C_{15}H_{31}C(O)-CCCCGGGEIKVAV-COOH$, Molecule 1, and $C_{15}H_{31}C(O)-CCCCGGGEIKVAV-NH_2$, Molecule 2.

[0022] **Figure 4** is a TEM micrograph of the negatively charged peptide-amphiphile nanofibers of molecule 1 self-assembled in the presence of KCl.

[0023] **Figure 5** is a schematic drawing of the chemical structure of the peptide-amphiphile $C_{15}H_{31}C(O)-CCCCRFEFREFR-NH_2$ illustrating the important groups of the molecule as well as a representation of the magnitude and direction of two of the amphiphilic moments in the molecule.

DETAILED DESCRIPTION OF THE INVENTION

[0024] As used throughout the specification, the following terms shall have the following meanings, unless the context clearly indicates otherwise. A nanofiber is defined as a cylindrical micelle comprising self-assembled peptide-amphiphiles. Examples of such nanofibers with a single species of peptide-amphiphile are described in *Science*, **294**, 1684, (2001). A nanofiber gel comprises a colloidal suspension of self assembled peptide-amphiphile nanofibers and a liquid. The nanofiber gel behaves as an elastic solid and retains its shape. Mineralization is a crystallization process used to describe the nucleation and growth of mineral crystals on the surface of a nanofiber or on the surfaces of nanofibers throughout a nanofiber gel.

[0025] Although the present invention will be described in considerable detail with respect to template mediated mineralization of hydroxyapatite on self assembled peptide-

amphiphile nanofibers of $C_{15}H_{31}C(O)-CCCCGGGS(P)RGD-COOH$, it is not intended to be limited to this system. Other materials, minerals, biominerals, magnetic materials, conductive materials, and crystals, for example: fluoroapatite, calcium oxalate, calcite, tin hydrogen phosphate, iron oxides, iron hydroxides, and various iron oxyhydroxides, (Fe_2O_3 , Fe_3O_4), TiO_2 , ZnO , and versions of these materials containing substitutions of the ions, vacancies, or interstitial ions, may be nucleated and grown by the practice of this invention. The invention is not limited by the size of the crystals or crystallites formed on the self assembled peptide-amphiphiles. The formed crystals may be semi-crystalline as well. Numerous positively and negatively charged peptide amphiphile species may be used in this invention, for example $C_{15}H_{31}C(O)-CCCCGGGS(A)RGD-COOH$, as well as those listed in Table 1 and Table 2. Although the present invention is described with respect to aqueous solutions, addition of other liquids or solvents like ethanol to the solution is not precluded in the practice of this invention. The invention may also be practiced by adding an effective amount of the peptide-amphiphile and salts as powders to a surgical site, for example, where fluids containing ions needed for gelation and mineralization may be found.

[0026] The peptide-amphiphiles and their self assembled nanofibers may promote adhesion and growth of cells on their surfaces. For example, the cell adhesion ligand RGD has been found in other contexts to play an important role in integrin-mediated cell adhesion. Peptide-amphiphile species with acidic amino acids and an amino acid with the RGD ligand could be used to mediate cell adhesion to the peptide-amphiphiles, their self assembled nanofibers, or nanofiber gels. The amino acid sequence IKVAV has been identified in other contexts as important for neuron growth and development. Accordingly, peptide-amphiphile species with acidic amino acids and the IKVAV sequence could be used in the practice of this invention to mediate neuron growth to the peptide-amphiphiles, their self assembled nanofibers, or nanofiber gels. The amino acid sequence YIGSR has been identified in other

contexts as important in for promoting cell-substrate adhesion among nerve cells also to play a role in axon guidance. Accordingly, peptide-amphiphile species with acidic amino acids and the YIGSR sequence could be used in the practice of this invention to promote cell-substrate adhesion among nerve cells to the peptide-amphiphiles, their self assembled nanofibers, or their nanofiber gels. For example in dentin, the phosphophoryn protein family contains numerous repeats of the amino acid sequences Asp-Ser(P)-Ser(P) and Ser(P)-Asp. These massively phosphorylated proteins are suspected to play an important role in hydroxyapatite mineralization. Accordingly, phosphoserine residues can be incorporated into the peptide sequence which, after self assembly, allows the fiber to display a highly phosphorylated surface equivalent to that presented by a long peptide segment. This, in part, captures the repetitive organization of phosphate groups found in phosphophoryn proteins.

[0027] In one embodiment a composition useful in the self assembly and mineralization of peptide-amphiphiles comprises a first solution of at least one negatively charged species of peptide-amphiphile and a soluble salt providing an anion of the mineral. The magnitude of the charges on the peptide amphiphile and anion do not have to be the same. The peptide-amphiphile is prepared using standard solid phase chemistry known to those skilled in the art. The dissolved anion may be obtained from a soluble salt or salts comprising the mineral. Alternatively, the mineral anion is formed by reaction known to those skilled in the art of the salts, for example with the pH adjustment of the solution, to yield the anion of the mineral. In cases where the mineral has more than one anion, a mixture of salts comprising the anions of the mineral may be used. In a preferred embodiment NaH_2PO_4 is the source of phosphate ion for the formation of hydroxyapatite. A second solution comprising one or more cations of the mineral obtained from soluble salt or salts is mixed with the first solution resulting in the self assembly of the peptide-amphiphiles into a nanofiber gel. The second solution may optionally contain one or more positively charged

peptide-amphiphiles. Templated mineralization of the cations and anions in the mixture occurs within the nanofiber gel formed from the peptide-amphiphiles.

[0028] In another embodiment, a composition useful in the self assembly and mineralization of peptide-amphiphiles comprises a first solution of at least one positively charged species of peptide-amphiphile and a soluble salt providing a cation of the mineral. The magnitude of the charges on the peptide amphiphile and cation do not have to be the same. The peptide-amphiphile is prepared using standard solid phase chemistry known to those skilled in the art. The dissolved cation may be obtained from a soluble salt or salts comprising the mineral. Alternatively, the mineral cation is formed by reaction of salts with the pH adjusted solution to yield the cation of the mineral. In cases where the mineral has more than one cation, a mixture of salts comprising the cations of the mineral may be used. A second solution comprising one or more anions of the mineral obtained from soluble salt or salts is mixed with the first solution resulting in the self assembly of the peptide-amphiphiles into a nanofiber gel. The second solution may optionally contain one or more negatively charged peptide-amphiphile. Templated mineralization of the cations and anions in the mixture occurs within the nanofiber gel formed by the peptide-amphiphiles.

[0029] In cases where the mineral has more than one cation, a mixture of salts comprising the cations may be mixed with the first solution to yield the homogeneous nanostructured material. The salt may be organic, inorganic, or a peptide-amphiphile. Examples of such cations obtained from salts and useful in the practice of this invention include but are not limited to NH_4^+ , Na^+ , Al^{+3} , Fe^{+3} , Mg^{+2} , Fe^{+2} , Ca^{+2} , Zn^{+2} , Cu^{+2} , Gd^{+3} and mixtures of these ions. Peptide amphiphiles with a positive charge may be considered as cations for the practice of this invention. Examples of anions useful in the practice of this invention include but are not limited to PO_4^{-3} , AsO_4^{-3} , CO_3^{-2} , OH^- , $\text{C}_2\text{O}_4^{-2}$ silicates, sulfates and mixtures of these and other anions known to those skilled in the art. Peptide amphiphiles

with a negative charge may also be considered as anions useful in the practice of this invention.

[0030] In another embodiment, the compositions can further comprise mixture of peptide-amphiphiles having the same signed ionic charge, but having different peptide sequences, functional groups, or magnitude of ionic charge. Acidic groups on poly-peptide substrates plays a key role in biomineralization processes. Phosphorylated groups are particularly preferred in this regard.

[0031] In another embodiment one or more of the peptide amphiphiles chelates an ion of the material to be formed. The chelating peptide-amphiphile may be neutral or ionically charged. The peptide amphiphile chelating the ion is then mixed with suitable ions or other peptides to form self-assembled nanofiber gels.

[0032] Notwithstanding embodiments provided above, broader aspects of the present invention include a peptide amphiphile composition having a hydrophobic or lyophobic component and a lyophilic peptide or peptide-like component. In various preferred embodiments, the hydrophobic component of such a composition is of sufficient length to provide amphiphilic behavior and micelle formation in water or another polar solvent system. Typically, such a component is a C₆ or greater hydrocarbon moiety, although other hydrophobic, hydrocarbon and/or alkyl components could be used as would be well-known to those skilled in the art to provide similar functional effect. Examples of such groups include but are not limited to arachidonyl, various length vinylic groups containing substituted with hydrogen or halogens such as fluorine, chlorine, bromine and iodine; acetylenic, diacetylenic and other acetylenic oligomers; various length alkene and isoprene groups substituted with hydrogen or halogens such as fluorine, chlorine, bromine and iodine. Regardless, the peptide component of such a composition can include the aforementioned RGD, IKVAV, or other sequences found especially useful for the nanofiber mineralization described herein.

[0033] Preferred peptide components of such compositions can also include a phosphoryl-functionalized residue or sequence, as described above. Inclusion of a phosphoserine residue has been found especially useful for hydroxyapatite mineralization. Other embodiments can include a phosphotyrosine residue. The peptide component of such compositions also include a residue or sequence capable of promoting intermolecular bonding and structural stability of the nanofibers available from such compositions. A sequence of cysteine residues can be used with good effect, providing for the facile intermolecular oxidation/reduction of the thiol functionalities.

[0034] Peptide components of this invention preferably comprise naturally-occurring amino acids. However, incorporation of artificial amino acids such as beta or gamma amino acids and those containing non-natural side chains, and/or other similar monomers such as hydroxyacids are also contemplated, with the effect that the corresponding component is peptide-like in this respect. Accordingly, such artificial amino acids, hydroxyacids or monomers can be used to meet the phosphorylation and/or intermolecular bonding objectives described above.

[0035] Various aspects of the present invention can be described with reference to the peptide amphiphile illustrated in Figure 1. Consistent with broader aspects of this invention, other peptide-amphiphiles, for example those listed in Table 1, may be used for the self-assembly of fibrous cylindrical micelles.

Table 1

| <u>PA</u> | <u>N-terminus</u> | <u>Peptide (N to C)</u> | <u>C-terminus</u> |
|-----------|-------------------|-------------------------|-------------------|
| 1 | C16 | CCCCGGGS(P)RGD | COOH |
| 2 | C16 | CCCCGGGS(P) | COOH |
| 3 | H | CCCCGGGS(P)RGD | COOH |
| 4 | C10 | CCCCGGGS(P)RGD | COOH |
| 5 | C6 | CCCCGGGS(P)RGD | COOH |
| 6 | C10 | GGGS(P)RGD | COOH |
| 7 | C16 | GGGS(P)RGD | COOH |
| 8 | C16 | AAAAGGGS(P)RGD | COOH |
| 9 | C10 | AAAAGGGS(P)RGD | COOH |
| 10 | C16 | CCCCGGGS(P)KGE | COOH |
| 11 | C10 | AAAAGGGS(P)KGE | COOH |
| 12 | C16 | AAAAGGGS(P)KGE | COOH |
| 13 | C22 | CCCCGGGS(P)RGD | COOH |
| 14 | C16 | CCCCGGGSRGD | COOH |
| 15 | C16 | CCCCGGGEIKVAV | COOH |
| 16 | C16 | CCCCGGGS(P)RGDS | COOH |
| 17 | C16 | CCCCGGGSS(P)D(S(P)D | COOH |

[0036] It should be noted that within the system examined, PAs 3 and 5 do not exhibit micelle formation, demonstrating a certain degree of hydrophobicity required for self-assembly of such compositions into the nanofibers of this invention. Depending upon desired cell or mineral growth, a phosphorylated moiety may not be required (see PAs 14 and 15). As discussed above, cellular adhesion or interaction is promoted by a particular sequence of the peptide components. With reference to PA's 10-12 and 15, a non-RGD sequence can be utilized depending upon cellular target. In particular, the IKVAV sequence has been identified in other contexts as important for neuron growth and development. Accordingly the amphiphile compositions of this invention can include a peptide component having such a sequence for corresponding use. Lastly, with respect to Table 1, it is noted that several PA compositions do not include cysteine residues: while such a peptide sequence can be used to enhance intermolecular nanofiber stability, it is not required for micelle formation in the first instance.

[0037] In part, the present invention also provides for a system including an aqueous solution of one or more of the amphiphile compositions described herein, and a factor or reagent sufficient to induce gelation under physiological conditions. Such gelation and/or self-assembly of various PA compositions into cylindrical micelle nanofibers can be achieved under substantially neutral pH conditions through drying, introduction of monovalent, divalent, or higher valency ions and/or the combination of differently charged amphiphiles. The approach of using differently charged amphiphiles can also be utilized to deliver in the self assembling nanofibrous system two or more bioactive molecules, each bearing different charges and this way combining the gelation technology with the delivery of multiple biological signals. Such facile factors, as described more fully below and in several of the following examples, can extend the system and/or methodology of this invention to a variety

of medical applications. These and other aspects of the present invention can be described with reference to the peptide-amphiphile, PA, compositions provided in Table 2, and with further reference to Figure 1 and Table 1.

| Table 2 | | | | |
|------------------|--------------------------|--------------------------------|--------------------------|---------------------------------|
| <u>PA</u> | <u>N-terminus</u> | <u>Peptide (N to C)</u> | <u>C-terminus</u> | <u>Net Charge at pH7</u> |
| 18 | C16 | CCCCGGGS(P)RGD | COOH | -3 |
| 19 | C16 | AAAAGGGGS(P)RGD | COOH | -3 |
| 20 | C10 | AAAAGGGGS(P)RGD | COOH | -3 |
| 21 | C16 | CCCCGGGSRGD | COOH | -1 |
| 22 | C16 | CCCCGGGEIKVAV | COOH | -1 |
| 23 | C16 | CCCCGGGKIKVAV | COOH | +1 |

[0038] In another embodiment of the invention, the degree of mineralization or crystallization is controlled. By modifying the degree of crystallization, control of the physical properties of the peptide-amphiphile mineral composite is achieved. The method comprises aging the mixture of the first and second solutions to control the extent of the mineralization and crystal growth reaction. Crystal growth requires, among other variables, control of the temperature and contact time of the mixture containing the cations and anions with the nanofiber gel.

[0039] As stated above, the amphiphile composition(s) of such a system may include a peptide component having residues capable of intermolecular cross-linking. The thiol moieties of cysteine residues can be used for intermolecular disulfide bond formation through introduction of a suitable oxidizing agent or under physiological conditions. Conversely such bonds can be cleaved by a reducing agent introduced into the system or under reducing conditions. The concentration of cysteine residues can also be varied to

control the chemical and/or biological stability of the nanofibrous system and therefore control the rate of therapeutic delivery or release of cells or other beneficial agent, using an effective amount of the nanofibers as the carriers. Furthermore, enzymes could be incorporated in the nanofibers to control biodegradation rate through hydrolysis of the disulfide bonds. Such degradation and/or the concentration of the cysteine residues can be utilized in a variety of tissue engineering contexts.

[0040] The ability of various peptide sequences in the peptide-amphiphiles to promote bone, tissue, or nerve growth may make systems of self assembled nanofibers useful in a number of different potential application. Specific applications include the delivery of therapeutics as well as biomedical and tissue engineering. As a self-supporting gel, it may have applications as a mineralizable bone-defect filler.

[0041] The assembly in the presence of biological ions such as Ca^{2+} may make the homogeneously mineralized material herein described particularly valuable for *in situ* and *in vivo* applications. It may also be used as a biological coating for orthopedic implants. These applications could find particularly valuable use in addressing medical problems such as osteooncology, congenital bone and tooth defects, osteoporosis, synthetic teeth, and dental implants.

[0042] The self-assembled peptide amphiphiles described in this disclosure are modifications of those originally described by Hartgerink, *et al.* (See e.g., J.D. Hartgerink, E. Beniash and S.I. Stupp, *Science* 294, 1683-1688, 2001), which is hereby incorporated in its entirety by reference thereto and the synthetic schemes set forth therein apply actually as well to the present invention.

[0043] Self-assembly and/or gelation under physiological conditions raises numerous implication regarding the end-use application and effect. A peptide-amphiphile mixture makes available a system for the formation of micellular nanofibers in an aqueous

environment at neutral and/or physiological pH conditions. Such a combination can be used to assemble nanofibers with a range of chemical groups or amino acids providing a variety of chemical or biological signals for corresponding cell adhesion, yielding enhanced properties with respect to tissue engineering or regenerative applications. It is contemplated that, alone or in conjunction with the other factors discussed herein, that preferred medical or therapeutic embodiments of such a system can be utilized. Furthermore, although the invention will be described in detail with respect to aqueous solution, the presence of non-aqueous liquids in the solution, like ethanol, will not limit the scope of the invention. Similarly, use of the terms hydrophobic and hydrophilic to describes the interaction of the multi-dimensional amphiphiles with water are construed to be equivalent to lyophobic and lyophilic for interaction of the multidimensional amphiphiles with non-aqueous liquids.

[0044] The present invention is directed to amphiphilic molecular compositions having more than one amphiphilic moment and also to supramolecular composition comprised of such amphiphilic molecules. An amphiphilic molecule with more than one amphiphilic moment is referred to as a multi-dimensional amphiphile. An example of such a molecule is shown schematically in Figure 5. The multi-dimensional amphiphilic molecule has a first chemical group or moiety, 1, covalently bonded to a second chemical group or moiety. In Figure 5 the second moiety is further divided into sections 2A and 2B. In Figure 5, and for illustrative purposes only, the second chemical moiety is a peptide comprised of amino acids. The amino acids may be, for example, naturally occurring amino acids, synthetic amino acids, β -amino acids, γ -amino acids, and or mixtures of these amino acids. The first and second moieties define a first amphiphilic moment of the amphiphilic molecule. The direction and magnitude of the first amphiphilic moment is along the axis of the molecule and is represented by divergent lines and labels lyophobic-1 and lyophilic-1 in Figure 5.

[0045] The second moiety of the molecule is further comprised of moieties covalently bonded together that define a second amphiphilic moment of the molecule. In Figure 5, for example, the second moiety is a peptide comprised of cysteine amino acids (2A) and polar and non-polar amino acids labeled 3, 4, 5, and 6. The amino acid moieties 4 and 5 are polar and substantially lyophilic because of the nature of the substituents. Examples of these substituents may be, acid groups, amine and amide groups, phosphate groups, hydroxyl groups, and sulfate groups, and carboxylic acid groups. The amino acid moieties 3 and 6 are the same in this example, but they may be different, and are non-polar and substantially lyophobic because of the nature of the substituents. Examples of these substituents may be phenyl, methyl, or substituted alkyl groups. The sequence of polar/lyophilic and non-polar/lyophobic moieties that make up the second lyophilic moiety in the molecule defines a second amphiphilic moment in the molecule. The second amphiphilic moment is not parallel to the first amphiphilic moment of the molecule. In Figure 5 the direction and magnitude of the second amphiphilic moment lies or is oriented across the first amphiphilic moment of the molecule; the two moments are not parallel to each other. The second amphiphilic moment is represented by the smaller divergent lines and labels lyophobic-2 and lyophilic-2 in Figure 5. A molecule with more than one amphiphilic moment is termed a multidimensional amphiphile; a molecule with more than one amphiphilic moment and having a peptide is termed a multidimensional peptide-amphiphile.

[0046] The portion of the peptide sequence labeled 2A in Figure 5 has cysteine amino acids capable of bonding together adjacent multidimensional peptide-amphiphiles in a self assembled nanofiber. In the molecule depicted in Figure 5, the cysteine amino acids may be replaced with glycine amino acids to provide flexibility to the peptide portion of the molecule. The cysteine amino acids may be replaced with other polar or non-polar amino acids. Other synthetic amino acids, β -amino acids, γ -amino acids with polar or non-polar

substituents may be used in the practice of this invention. Incorporation monomers such as hydroxyacids are also contemplated, with the effect that the corresponding component is peptide-like in this respect. The sequence of alternating polar and non-polar moieties and more specifically the alternating polar and non-polar amino acids may be varied and the invention is not limited to disclosed combinations. The peptide may contain the amino acid sequence IKVAV. Other examples of peptides with alternating hydrophobic and hydrophilic amino acids, include but are not limited to: YQYQYQ; AQAQAAQ; YQAQYQAQ; RADARADA; HNHNNHN; HNHQHNNQH.

[0047] This invention is more specifically directed to the synthesis of peptide-amphiphiles with more than one amphiphilic moment and to supramolecular compositions comprised of such multi-dimensional peptide-amphiphiles. These supramolecular compositions can be formed by self assembly of multi-dimensional peptide-amphiphiles by mixing them with a solution comprising a cation. In a preferred embodiment monovalent cations are used to induce self assembly of the multidimensional peptide-amphiphiles. Examples of such cations include but are not limited to Na^+ , K^+ , or RNH_3^+ , where R is a hydrogen, a phenyl group, or an alkyl group.

[0048] In a preferred embodiment of the invention, a supramolecular composition is formed by mixing multi-dimensional peptide-amphiphiles containing the IKVAV amino acid sequence with a monovalent cation from salts such as NaCl and KCl. Examples of suitable multi-dimensional peptide amphiphiles are Molecule 1 and Molecule 2 illustrated in Figure 3. The peptide-amphiphile has amino acids with moieties for covalent coupling. Examples of such amino acids include but are not limited to cysteine. The peptide-amphiphile also has amino acids that provide a flexible linkage within the peptide portion of the molecule. Examples of such amino acid moieties include but are not limited to glycine.

[0049] More specifically, the peptide-amphiphiles of this invention contain a hydrophobic or lyophobic component of sufficient length to provide amphiphilic behavior and micelle formation in water or polar solutions. Typically, such a first moiety is a C₆ or greater hydrocarbon group, although other hydrocarbon and/or alkyl components could be used in place of or bonded as a substituents onto the hydrocarbon group as would be well known to those skilled in the art. Examples of such groups include but are not limited to arachidonyl, various length vinylic groups containing substituted with hydrogen or halogens such as fluorine, chlorine, bromine and iodine; acetylenic, diacetylenic and other acetylenic oligomers; various length alkene and isoprene groups substituted with hydrogen or halogens such as fluorine, chlorine, bromine and iodine.

[0050] The invention may also be practiced by adding an effective amount of the peptide-amphiphile and salts as powders to a surgical site, for example, where fluids containing ions needed for gelation and mineralization may be found.

[0051] The self assembly and gelation of peptide-amphiphiles like Molecule1 and Molecule 2 to form the supramolecular composition is triggered by addition of monovalent cations into the peptide-amphiphile solution. The monovalent salts provide an ionic environment that is believed to reduce the electrostatic repulsive force between peptide-amphiphiles of the same polarity. Examples of suitable monovalent cations include but are not limited to Na⁺, K⁺, or RNH₃⁺. The monovalent cations in solution enable the peptide-amphiphiles to establish short range hydrophobic interactions between the aliphatic tails of the molecules as well as the amphiphilic portions of the peptide sequence. Amphiphilic peptides were previously reported to self assemble into β -sheet based supramolecular structures (Aggeli et al 1977, and Holmes et al., 2000).

[0052] One advantage of the present invention is that the peptide amphiphiles self assemble to form fibers rather hollow tubes. Such fibers may be suitable for deliver or

encapsulation of various cell therapies and provide close surfaces for templated tissue, bone, or nerve growth. The delivery of an effective amount of such encapsulated therapeutics to a patient may be useful in the treatment of a variety of conditions. The structure of the peptide-amphiphile may be changed to create self assembled structures having various pore sizes. Although the present invention will be described in considerable detail with respect to self assembly of multi-dimensional peptide amphiphiles with the IKVAV peptide and their use in promoting cell growth, it is not intended to be limited to this amino acid sequence or to cell growth. Other multi-dimensional peptide amphiphiles with alternating polar and non-polar amino acids sequences may self assemble and direct the growth of tissues, materials, minerals, biominerals, magnetic materials, conductive and semiconductor materials, and crystals on their surfaces. Examples of such materials include but are not limited to fluoroapatite, calcium oxalate, calcite, tin hydrogen phosphate, iron oxides, iron hydroxides, and various iron oxyhydroxides, (Fe_2O_3 , Fe_3O_4), TiO_2 , ZnO . Versions of these materials containing substitutions of the ions, vacancies, or interstitial ions, may also be nucleated and grown by the practice of this invention. The invention is not limited by the size of the crystals or crystallites formed on the self assembled peptide-amphiphiles. The formed crystals may be semi-crystalline as well.

[0053] Another difference between the peptide-amphiphiles in the present invention from known amphiphilic molecules is that the present invention's peptide-amphiphiles are two-dimensional amphiphiles. The peptide-amphiphiles of the present invention have two "amphiphilic moments" oriented in different directions. One amphiphilic moment coincides with or is parallel to the backbone axis of the molecule, the second amphiphilic moment is not parallel to the backbone of the molecule and is directed across the peptide sequence of the molecule. The alkyl tail moiety of the peptide-amphiphile is much more hydrophobic than any moieties on the amino acids composing the peptide part of the peptide-amphiphile. The

amphiphilic moment along the backbone of the peptide-amphiphile molecule is much stronger than the amphiphilicity across the IKVAV segment. The amphiphilicity in different directions is different; it is much stronger along the backbone of the molecule than along the sides of the amphiphilic peptide segment. This molecular design may serve as a prototype for other multi-dimensional amphiphilic molecules, which may not include the peptide or alkyl moieties. In principle any molecule with two or more axes of amphiphilicity may be described as a multi-dimensional amphiphile. Multi-dimensional amphiphiles can serve as the building blocks for supramolecular assemblies and lead to the development of new supramolecular structures that may find application in different fields of nanotechnology and biomedical applications.

[0054] Supramolecular compositions formed from self assembled multi-dimensional amphiphiles may be administered to treat a patient. For example, the patient may require assistance stimulating cell or nerve growth. The treatment comprises administering a multi-dimensional peptide-amphiphile composition having a cell growth peptide sequence within the peptide-amphiphile to a site on the patient requiring treatment. The supramolecular composition may form using sodium or potassium ions already present in the patient. Alternatively a separate solution containing monvalent ions may be administered to the patient to cause the formation of the supramolecular composition from the multi-dimensional peptide-amphiphile solution.

[0055] Synthetic supramolecular assemblies could also be designed and synthesized with channels or pores for targeted delivery of drugs to specific cells or organs. These supramolecular assemblies may provide for encapsulation of materials and molecules such as therapeutic drugs, cell therapies, cancer treatments, antibodies, magnetic colloids, conductive colloids, carbon nanotubes, and semiconductor colloids.

EXAMPLE 1

[0056] This example illustrates the components of a liquid composition and its use to form homogeneously distributed, and directionally orientated hydroxyapatite crystals within a nanofiber gel comprised of self assembled peptide-amphiphiles.

[0057] Peptide amphiphile $C_{15}H_{31}C(O)-CCCCGGGS(P)RGD-COOH$ (1) was prepared using standard solid phase chemistry; its structure is shown in Figure 1.

[0058] A liquid composition for the self assembly of (1) and mineralization of hydroxyapatite was prepared by dissolving 20 millimoles NaH_2PO_4 (Aldrich) into 200 microliters of (1) at a concentration of 10 mg/ml in water. The pH of the solution was adjusted to 7.7.

[0059] The liquid composition containing (1) and NaH_2PO_4 was mixed with 40 micromoles of $CaCl_2$ (Aldrich).

[0060] Self assembled gel comprising (1) was formed immediately upon addition of $CaCl_2$ to the liquid composition of the peptide-amphiphile (1) preloaded with a source phosphate anions from the NaH_2PO_4 . The self assembled gel was initially transparent but turned a white color after about 2 hours suggesting that the mineralization process had started. Samples of the gel after 2 hours, 1 day, and 5 days were mounted on carbon-coated TEM grids. TEM studies of the 1 and 5 day samples show a multi-crystalline material composed of plate like crystals~ 5 nm thick and 50 to 100 nm long. The plate like crystals were similar to those observed in the mineralization experiments on the pre-assembled template. Electron diffraction of the material matches the diffraction pattern for hydroxyapatite. Both TEM images of the mineralized gel as well as diffraction patterns suggest local orientation of the hydroxyapatite crystals in the self assembled gel. These data suggest that the c-axis of the hydroxyapatite crystals is co-aligned with the nanofiber axis as

shown in Figure 2. This observation suggests that the peptide-amphiphile nanofibrils control nucleation and direction of the crystal growth.

[0061] The results of this example show that peptide-amphiphile organo-mineral composite materials may be manufactured in one step by adding metal ions to a liquid composition of peptide-amphiphile pre-loaded with a source of phosphate anions. The example further illustrates that the self-assembly of the peptide-amphiphiles occurs upon addition of a metal ion and that they later serve as a template for the directed mineralization of hydroxyapatite. This example further illustrates that the method for making the hydroxyapatite composites is useful for preparing homogenous nanostructured composite materials.

EXAMPLE 2

[0062] In another example of this invention, the composition and method consists of using highly charged peptide amphiphile species (16carbon alkyl tail with a sequence like CCCCCGGSS(P)DS(P)D with a -7 charge, for example) dissolved in a solution of negative ions (phosphate ions with a -3 charge), call this solution X. A second solution with a positively-charged peptide amphiphile species (such as 16 carbon alkyl tail with a sequence like ACAAGGGKRGDS - an amine terminated PA at +1 charge) in a solution with positively-charged ions, such as Ca^{2+} ; call this solution Y. In both solutions the peptide heads are charged and the structural element of the peptide can be varied, to give different charged peptide-amphiphile species, depending on the application.

[0063] The positive and negative peptide amphiphiles alone (no added salt ions) will gel each other, reaction 1, when mixed in the right ionic ratios (1:7, (-):(+) in this instance), forming mixed peptide amphiphile nanofibers, theoretically composed of 7 positive peptide amphiphiles for every 1 of the negative peptide amphiphiles. The positive peptide amphiphile solution Y may be gelled, reaction 2, with the negative ions (for example a

solution containing phosphate ion PO_4^{-3}). The negative peptide amphiphile solution X will be gelled, reaction 3, with positive ions (for example a solution containing Ca^{+2}). The positive peptide amphiphile does not gel, reaction 4, in positive ions (for example Ca^{+2}). The negative PA does not gel, reaction 5, in negative ions (for example phosphate ion PO_4^{-3}). Mixing the positive and negative ions (calcium cation with phosphate anion) will make calcium phosphate mineral (reaction 6). When solution X is mixed with solution Y, a gel forms, reaction 7, very quickly. It is believed that mineral (calcium phosphates and possibly sodium chloride) is nucleated and grown intimately and substantially throughout the mixed-peptide amphiphile fibers. The gel formed may be the product of reactions 1, 2, 3, and 6, occurring in approximately the same time frame. The combination allows us formation of a mineralized gel at physiologic pH. This example further demonstrates that by using two distinct peptide-amphiphiles, different peptide sequences which might work well in concert with one-another (such as IKVAV and YIGSR) might be simultaneously combined during the assembly and mineralization process.

EXAMPLE 3

[0064] This example describes the synthesis of peptide-amphiphiles with more than one amphiphilic moment, and describes the synthesis of a supramolecular composition comprised of self-assembled multi-dimensional peptide-amphiphiles. A supramolecular composition is formed by combining multi-dimensional amphiphiles containing the IKVAV amino acid sequence with monovalent salts such as NaCl and KCl.

[0065] Molecule 1, as shown in Figure 3, is a peptide-amphiphile that contains the amino acid sequence IKVAV moiety with terminal end group $-\text{COOH}$; this sequence has been shown to promote axon outgrowth in neurons. Molecule 2, also shown in Figure 3, is a peptide-amphiphile that contains the sequence IKVAV moiety with the terminal end group $-\text{NH}_2$, which has similarly been shown to promote axon outgrowth in neurons. The two

molecules dissolve in pH 7.5-adjusted water at a concentration of about 10mg/mL. Molecule 1 has a charge of (-1) and molecule 2 has a charge of (+2) under these conditions. A self-supporting gel forms on mixing of either of the peptide-amphiphile solutions with 200 mM KCl or NaCl solutions. Examination of the gels formed by these reactions by negative stain TEM shows that the gels are composed of nanofibers of the self assembled peptide-amphiphiles.

[0066] In all cases self assembled gels comprised of the nanofibers were formed similar to those described elsewhere (Hartgerink et al., 2001; Hartgerink et al., 2002). In contrast no self assembly or gel formation was observed when other negatively or positively charged peptide-amphiphiles were exposed to the NaCl or the KCl at concentrations up to 6 M. The fact that molecules 1 and 2 assemble in the presence of the monovalent salts sets them apart from the other molecules studied. Experiments with negatively charged molecules that do not contain amphiphilic peptide sequences show that the charge screening by monovalent inorganic ions alone is not sufficient to induce peptide-amphiphile self-assembly. The reason for this difference may be in the structure of these molecules. Both these molecules contain IKVAV sequence at the c-terminus of the peptide segment. This sequence is comprised of alternating extremely hydrophobic amino acids I and V and more hydrophilic ones such as A and K. Since the side chains of adjacent amino acids are located on opposite sides of the peptide backbone, this sequence is amphiphilic. The molecules 1 and 2 may be considered as double or two dimensional amphiphiles; one moment of amphiphilicity coinciding with the backbone axis of the molecule and amphiphilic peptide segment at c-terminus, and the second moment of amphiphilicity directed across the amphiphilic peptide segment. Previously amphiphilic peptides have been shown to assemble into ribbon like structures forming 3-D networks upon addition of monovalent salts (Zhang et al., 1995; Caplan et al., 2000). It was suggested that the function of inorganic ions in these

systems is to screen charged functional groups of the peptide that facilitates supramolecular assembly of amphiphilic peptides. It is believed that a similar mechanism is involved in the self assembly of peptide-amphiphiles containing IKVAV sequences in addition to the hydrophobic interactions between the alkyl parts of the molecules.

[0067] *Materials and Methods:* Abbreviations: PA: peptide-amphiphile, TEM: transmission electron microscopy.

[0068] *Chemicals:* Except as noted below, all chemicals were purchased from Fisher or Aldrich and used as provided. Amino acid derivatives were purchased from Applied BioSystems and NovaBiochem; derivatized resins and HBTU were also purchased from NovaBiochem. All water used was deionized with a Millipore Milli-Q water purifier operating at a resistance of 18 MW.

[0069] *Synthesis of the peptide-amphiphiles:* The peptide-amphiphiles were prepared on a 0.25mmole scale using standard Fmoc chemistry on an Applied Biosystems 733A automated peptide synthesizer. After the peptide portion of the molecules was prepared, the resin was removed from the automated synthesizer and the N-terminus capped with a fatty acid containing 16 carbon atoms. The alkylation reaction was accomplished using 2 equivalents of the fatty acid, 2 equivalents HBTU and 6 equivalents of DiEA in DMF. The reaction was allowed to proceed for at least six hours after which the reaction was monitored by ninhydrin. The alkylation reaction was repeated until the ninhydrin test was negative. Only two couplings were required in each case.

[0070] Cleavage and deprotection of the molecules was accomplished with a mixture of TFA and TIS in a ratio of 95:5 for three hours at room temperature. The cleavage mixture and two subsequent TFA washings were filtered into a round bottom flask. The solution was roto-evaporated to a thick viscous solution. This solution was triturated with cold diethylether. The white precipitate was collected by filtration, washed with copious cold

ether and dried under vacuum. The molecules were then dissolved in water at a concentration of 10mg/mL, adjusting the pH to improve solubility. The solution was initially acidic in both cases. In the case of molecule 1, the pH was raised to about pH 8 with 2M and 100mM KOH, then back-titrated to pH 7. In the case of molecule 2, the molecule was most soluble at low pH, but remained in solution when the pH was raised to 7 using KOH. The molecules were characterized by ESI MS and were found to have the expected molecular weight.

[0071] Transmission Electron Micrographs of samples of the supramolecular compositions from the multi-dimensional peptide-amphiphiles, Molecule 1 and Molecule 2, were prepared as follows. A small sample of the supramolecular composition gel, prepared in bulk as described above, was smeared onto a holey carbon coated TEM grid (Quantifoil). Negative staining with PTA (phosphotungstic acid) was used in this study[Harris, 1991 #93]. In all cases electron microscopy was performed at an accelerating voltage of 200kV.

[0072] Various other amphiphile compositions of this invention can be prepared in analogous fashion, as would be known to those skilled in the art and aware thereof, using known procedures and synthetic techniques or straight-forward modifications thereof depending upon a desired amphiphile composition or peptide sequence.

[0073] All of the embodiments disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or

similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

[0074] While the principles of this invention have been described in connection with specific embodiments, it should be understood clearly that these descriptions are added only by way of example and are not intended to limit, in any way, the scope of this invention. For instance, various peptide amphiphiles have been described in conjunction with specific residues and corresponding cell adhesion, but other residues can be used herewith to promote a particular cell adhesion and tissue growth on the nanostructures prepared therefrom. Likewise, while the present invention has been described as applicable to biometric material or tissue engineering, it is also contemplated that gels or related systems of such peptide amphiphiles can be used as a delivery platform or carrier for drugs, cells or other cellular or therapeutic material incorporated therein. Other advantages and features will become apparent from the claims filed hereafter, with the scope of such claims to be determined by their reasonable equivalents, as would be understood by those skilled in the art.

REFERENCES

- Aizenberg, J.; Black, A. J.; Whitesides, G. M. *Nature*, **398**, 495-498, (1999).
- Braun, P. V.; Stupp, S. I. *Materials Research Bulletin*, **34**, 463-469, (1999).
- Hartgerink et al; *Science*, **294**, 1684-1688, (2001).
- Hartgerink et al, *PNAS*, **99(8)**, 5133-5138, (2002).
- Preparation of self-assembling amphiphile for construction of peptide secondary structures. G. B. Fields, M. V. Tirrell, United States Patent Number **6,096,863**.
- M. R. Caplan, P. N. Moore, S. G. Shang, R. D. Kamm, D. A. Lauffenburger, *Biomacromolecules*; **1**, 627, (2000).

A. L. Litvin, S. Valiyaveetil, D. L. Kaplan Process for nucleation of ceramics and products thereof. United States Patent Number **5,993,541**

Xu, G. F. et al, *Journal of the American Chemical Society* **120**, 11977-11985, (1998)